Melanoma Inhibitor of Apoptosis Protein (ML-IAP) Specific Cytotoxic T Lymphocytes Cross-React with an Epitope from the Auto-Antigen SS56

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A large proportion of melanoma patients host a spontaneous T-cell response specifically against ML-IAP-derived peptides. In this study, we describe that some ML-IAP-specific cytotoxic T cells isolated from melanoma patients cross react with an epitope from the auto-antigen SS56. SS56 is a recently described target of autoantibody responses in Sjögren's syndrome (SS) as well as systemic lupus erythematosus (SLE). Here, we describe that SS56 is also an auto-antigen for T cells in SS and SLE. Hence, SS56-specific T cells could readily be detected in circulation and among the infiltrating cells of SLE skin lesions. SS56-specific T cells were able to lyse target cells presenting the peptide epitope on the surface. Notably, SS56-specific CD8 T cells isolated from an SS patient cross reacted with the ML-IAP epitope. This early evidence of a target for auto-reactive CTL in SS and SLE patients; it is to our knowledge previously unreported and underscores the important role of CD8 T cells in autoimmune disorders. Furthermore, the cross-reactivity against the auto-antigen SS56 and the tumor-antigen ML-IAP confirms the link between autoimmunity and anti-cancer cellular immune responses.

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INTRODUCTION

A finely orchestrated balance between activating and inhibitory signals is fundamental for the ability of the immune system to effectively attack and eliminate pathogenic microbes without reacting against self-antigens. Derangements of this balance underlie the pathogenesis of autoimmune diseases (Blanco *et al.*, 2005b). The majority of research into the biology of autoimmune diseases has—historically—focused on B-lymphocytes and CD4 T-helper cells. However, recent technical advances as well as an improved understanding of the regulatory role have brought more attention to CD8 T cells (Yasuoka *et al.*, 2004; Sospedra and Martin, 2005; Andersen *et al.*, 2006). It was recently reported that a high proportion of activated CD8 T cells could be found in the blood of systemic lupus erythematosus (SLE) patients (Blanco *et al.*, 2005a). Furthermore, CD8 T cells infiltrate the periglomerular area in patients with severe lupus nephritis and are linked to a poor outcome after induction therapy (Couzi *et al.*, 2007).

The close association between tumor immunity and auto immunity is underlined by many observations, for example, the association between onset of vitiligo and better outcome of melanoma (Bystryn *et al.*, 1987), as well as the correlation between autoimmunity and survival during treatment of melanoma patients with interferon-α (Gogas *et al.*, 2006). In addition, treatment with anti-CTLA-4 antibodies can induce durable objective clinical responses in melanoma and renal cell carcinoma patients, who are directly correlated with immune-related adverse events (Beck *et al.*, 2006; Kaufman and Wolchok, 2006; Downey *et al.*, 2007). Conversely, increased knowledge on the parameters that control and inhibit immune responses may provide strategies for enhancement of the efficacy of anti-cancer immunotherapy. Hence, tolerance to tumor antigens can be mediated through the same mechanisms that induce T-cell tolerance to normal self-antigens to avoid autoimmunity (Yan and Mamula, 2002).

Melanoma inhibitor of apoptosis protein (ML-IAP, also named livin) is a critical cellular factor as increased expression levels confer resistance to apoptotic stimuli, thereby contributing to the pathogenesis and progression of melanoma (Kasof and Gomes, 2001; Nachmias *et al.*, 2003). The majority of melanoma cell lines as well as melanoma cultures from primary melanoma patients express high levels of ML-IAP in contrast to primary melanocytes. We and others have identified peptide-epitopes derived from ML-IAP, which are recognized by circulating cytotoxic T lymphocytes (CTL) obtained from melanoma patients (Schmollinger *et al.*, 2003;

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Abbreviations: CTL, cytotoxic T lymphocytes; ML-IAP, melanoma inhibitor of apoptosis protein; PBL, peripheral blood lymphocytes; pSS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus; TCR, T-cell receptors

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Andersen *et al.*, 2004a, b). Surprisingly, we also identified a T-cell response against an HLA-A2-restricted peptide ML-IAP$_{280-289}$ (QLCPICRAPV) in a few control donors (Andersen *et al.*, 2004b). Consequently, we examined if any homolog peptides were present in other human proteins, particularly those associated with autoimmune diseases. To this end, we identified a highly homologous peptide from the protein SS56 (SS56$_{55-64}$ (YTCPLCRAPV)); SS56 is a recently described cellular target of antibody responses in Sjögren's syndrome (SS) and SLE (Billaut-Mulot *et al.*, 2001). The major target organs of SS are lacrimal glands and salivary glands where prominent lymphocytic infiltration occurs. Extraglandular manifestations and varying levels of organ-unspecific autoantibody production indicate the systemic nature of this disease (Nakamura *et al.*, 2006). SLE is also a systemic autoimmune disease predominantly affecting women, with a wide range of clinical features and a prognosis that may range from benign to fatal, primarily depending on the extent and severity of internal organ involvement such as nephritis and central nervous system involvement (Cook and Botto, 2006; D'Cruz *et al.*, 2007).

The purpose of this study was to investigate whether SS56 is a target for CD8 T cells in primary SS (pSS) and SLE. Furthermore, we examined if cytotoxic T cells cross-react between SS56 and ML-IAP.

RESULTS

ML-IAP$_{280-289}$-specific T cells cross react with SS56$_{55-64}$

ML-IAP$_{280-289}$-specific T-cell cultures were established using peripheral blood lymphocytes (PBL) from a melanoma patient harboring a spontaneous T-cell response against the peptide as identified by ELISPOT assay (Andersen *et al.*, 2004b). PBL from this melanoma patient were stimulated four times *in vitro* with ML-IAP$_{280-289}$-pulsed autologous dendritic cells. The T-cell culture was examined for recognition of ML-IAP$_{280-289}$ as well as SS56$_{55-64}$ by IFN-γ ELSPOT assay. A strong T-cell response was observed against ML-IAP$_{280-289}$, as well as a minor response against SS56$_{55-64}$ (Figure 1a). Next, CTL clones were established from the ML-IAP$_{280-28}$-specific bulk culture by limiting dilution. After a short expansion step, the specificity of the growing clones was analyzed in standard ^{51}Cr-release assays. Hence, a panel of the ML-IAP$_{280-289}$-specific T-cell clones were examined for recognition of ML-IAP$_{280-289}$ as well as SS56$_{55-64}$ using peptide-pulsed T2-cells as target cells. Whereas some clones only recognized the ML-IAP$_{280-289}$ peptide, other clones in addition lysed T2-cells pulsed with the SS56$_{55-64}$ peptide (Figure 1b). Next, we examined the T-cell avidity of the cross-reactive clones against ML-IAP$_{280-289}$ as well as SS56$_{55-64}$. T-cell avidity can be defined as the lowest amount of peptide needed for the activation of the T cells. We found that the amount of peptide needed for activation was lower for ML-IAP$_{280-289}$ compared to that of SS56$_{55-64}$ (Figure 1c).

FACS analyses of SS56-specific T cells

The spontaneous occurrence of SS56$_{55-64}$-specific CTL in PBL from pSS and SLE patients was evaluated using FACS analyses and HLA-A2/SS56$_{55-64}$ dextramer staining. These data confirmed that SS56$_{55-64}$-specific CD8 T cells are present in the blood of HLA-A2 pSS patients and SLE patients (Figure 2). Figure 2a illustrates an example of a T-cell response in a pSS and an SLE patient with a HLA-A2/HIV-1

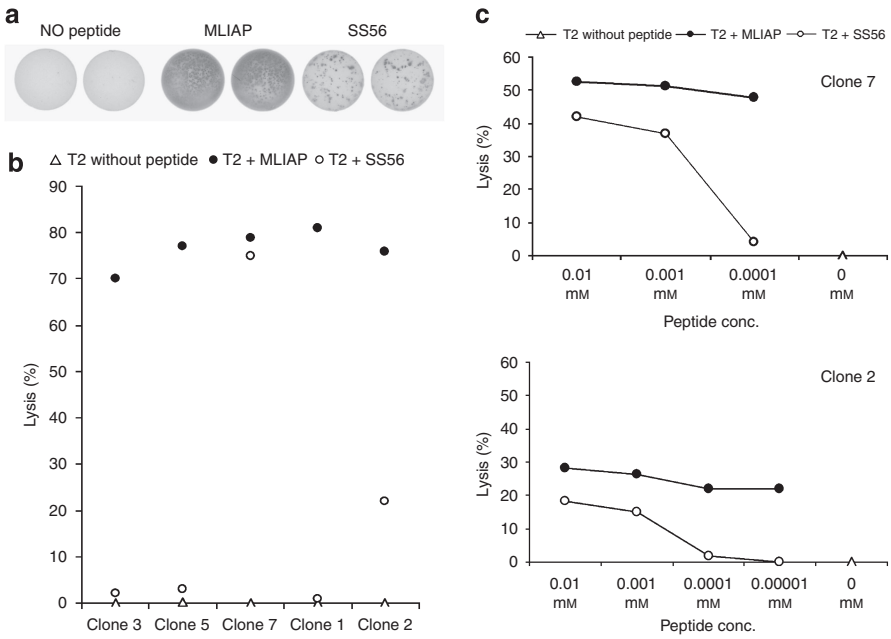

Figure 1. ML-IAP$_{280-289}$-specific T cells recognize SS56$_{55-64}$. (**a**) Specificity of an ML-IAP$_{280-289}$-specific T-cell culture measured in an IFN-γ ELISPOT assay. T cells were plated at 10^4 cells per well in duplicates either without peptide or with ML-IAP$_{280-289}$ or SS56$_{55-64}$ peptide. (**b**) Specificity of five ML-IAP$_{280-289}$-specific T-cell clones assayed by ^{51}Cr-release assays measuring cell lysis of T2 cells without peptide or pulsed with ML-IAP$_{280-289}$ or SS56$_{55-64}$ peptide (E/T ratio = 15:1). (**c**) Peptide affinity of two ML-IAP$_{280-289}$-specific T-cell clones assayed by ^{51}Cr-release assays measuring cell lysis of T2 cells pulsed with different concentrations of the ML-IAP$_{280-289}$ or SS56$_{55-64}$ peptide (E/T ratio = 10:1).

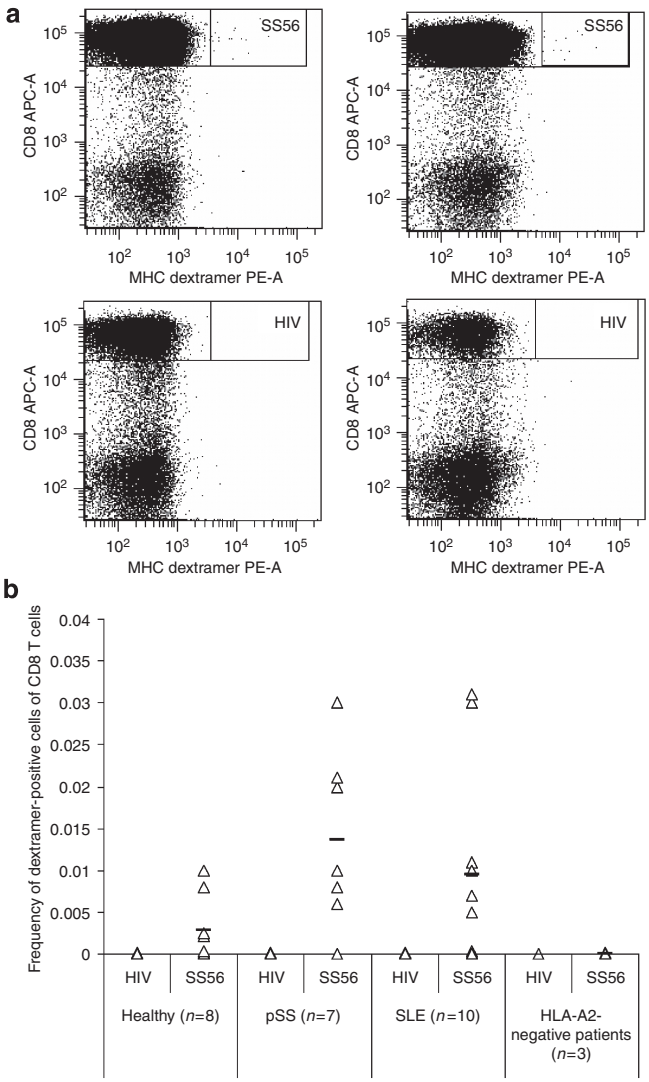

Figure 2. Detection of SS56 specific CD8 T cells in peripheral blood from patients with pSS and SLE. (**a**) Example of $SS56_{55-64}$-specific CD8 T cells in PBL from a patient with pSS (left) and a patient with SLE (right) visualized by flow cytometry using the dextramer complex HLA-A2/ $SS56_{55-64}$-PE, and CD8-allophycocyanin. As a negative control, PBL from the same patients were stained with the dextramer complex HLA-A2/HIV $pol_{476-484}$-PE, and CD8-allophycocyanin. (**b**) The frequency of HIV-1 $pol_{476-484}$ and $SS56_{55-64}$ dextramer-positive CD8 T cells in PBL from eight HLA-A2-positive healthy individuals, seven HLA-A2-positive patients with pSS, 10 HLA-A2-positive patients with SLE and three HLA-A2-negative patients.

$pol_{476-484}$ dextramer used as control. PBL from eight HLA-A2-positive healthy individuals, seven HLA-A2-positive patients with pSS, and 10 patients with SLE were analyzed. This analysis revealed an average frequency of SS56-specific cells of around 0.01% of the CD8 cells in PBL from pSS and SLE patients (Figure 2b), with a maximum of 0.03% in one pSS patient and two SLE patients. As negative controls we included HLA-A2-negative pSS and SLE patients. No specific cells could be detected in any of these patients (Figure 2b).

Detection of SS56 reactive T cells in active SLE skin lesions

The prognostic and clinical significance of measuring specific T cells in peripheral blood has been questioned repeatedly

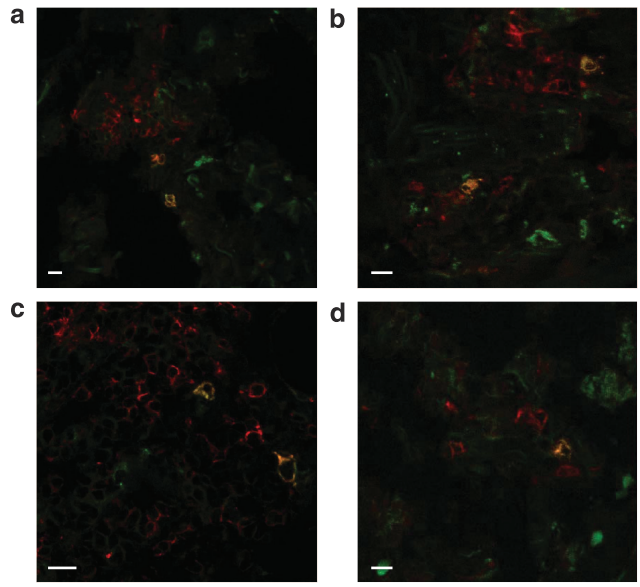

Figure 3. *In situ* detection of SS56-reactive CTL. Confocal laser scanning microscopy was used to detect the presence of CTL reacting with a Cy3-conjugated anti-CD8 antibody (red channel) and an FITC-conjugated multimeric HLA-A2/ $SS56_{55-64}$ construct (green channel) in skin lesions from HLA-A2-positive and HLA-A2-negative SLE patients. Double positive cells appear yellow. HLA-A2/$SS56_{55-64}$-reactive CD8 T cells were detected in 2 out of 10 HLA-A2 patients. Two respective areas of these tissue samples are depicted; **a** and **b** derived from one, **c** and **d** from the other patient. Magnification **a**, 400×; **b**–**d** 600×. The scale bar represents 25 μm.

(thor Straten *et al.*, 1999). Thus, we also tested for the presence of HLA-A2/$SS56_{55-64}$ reactive CD8 T lymphocytes among infiltrating lymphocytes in inflammatory skin lesions of SLE patients by means of HLA/peptide dextramer staining. FITC-conjugated HLA-A2/ $SS56_{55-64}$ dextramers were used to stain acetone-fixed, frozen material as described previously (Andersen *et al.*, 2001b; Schrama *et al.*, 2002), and antigen-specific cells were visualized using a confocal laser microscope. Sections of cutaneous SLE manifestations from 10 HLA-A2-positive and 10 HLA-A2-negative SLE patients were analyzed. This analysis revealed the *in situ* presence of $SS56_{55-64}$-reactive, CD8 CTL in two of the HLAA2-positive, but none of the HLA-A2-negative patients (Figure 3). Tissue samples obtained from HLA-A2-positive patients suffering from allergic contact dermatitis and atopic eczema served as controls; in none of these lesions could $SS56_{55-64}$-reactive, CD8 CTL be detected. It should be noted, that only the CD8/multimer double-positive cells were regarded as a positive signal—not specific staining of collagen fibers by FITC, which is a well known problem; the difference in morphology between collagen and cells is a valid tool to distinguish them from each other.

Functional T-cell responses against the SS56-derived peptide in PBL from patients with pSS, SLE patients, and healthy individuals

By means of the ELISPOT assay, we further examined peripheral blood T cells from HLA-A2 patients with pSS

($n=8$) and SLE ($n=11$), as well as healthy individuals ($n=11$) for $SS56_{55-64}$ peptide-specific IFN-γ production. This method was previously demonstrated to be highly effective for identifying antigen-specific CTL in cancer patients (Herr *et al.*, 1999; Andersen *et al.*, 2001a; Scheibenbogen *et al.*, 2002). PBL were stimulated once with peptide *in vitro* before examination by ELISPOT. This procedure was chosen to extend the sensitivity of the ELISPOT as described (Andersen *et al.*, 2001a; Keilholz *et al.*, 2002). Using this approach, we were able to identify more than 50 $SS56_{55-64}$-specific T cells/2×10^5 PBL in five pSS-patients and three SLE patients (Figure 4). To further characterize the functional capacity of SS56-reactive CTL, these cells were isolated from PBL from a pSS patient by FACS using HLA-A2/ $SS56_{55-64}$ dextramer complexes. After expansion using phytohemagglutinin, IL-2, and feeder cells, we examined the lytic capacity of these cells by chrome release assay. This revealed that SS56-specific T cells indeed killed $SS56_{55-64}$ loaded T2 cells. In addition, these isolated $SS56_{55-64}$-reactive T cells lysed ML-$IAP_{280-289}$ loaded T2 cells, demonstrating the cross-reactivity of the T cells (Figure 5).

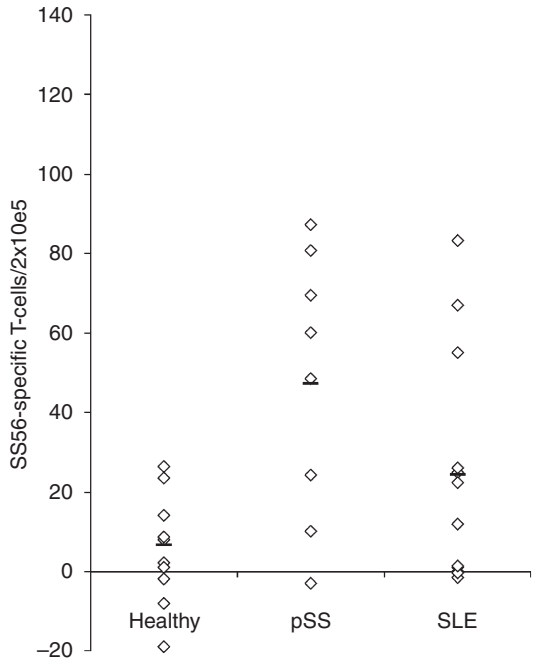

Figure 4. HLA-A2-restricted T-cell responses against SS56 as measured by INF-γ ELISPOT. PBL from 11 healthy individuals, eight patients with pSS, and 11 patients with SLE were analyzed. All individuals were HLA-A2 positive. T lymphocytes were stimulated once with $SS56_{55-64}$ (YTCPLCRAPV) before being plated at 3×10^5 cells per well in duplicates either without or with peptide. The average number of peptide-specific spots (after subtraction of spots without added peptide) was calculated for each patient using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC, Cleveland).

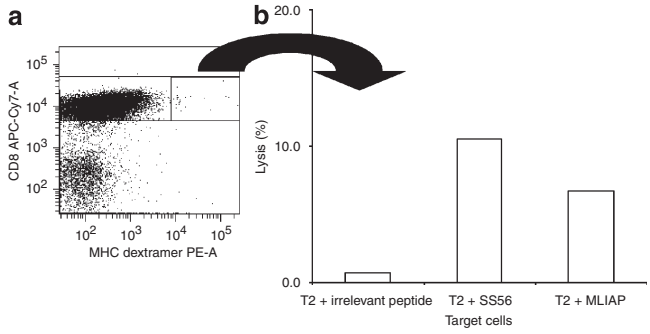

Figure 5. Cytolytic capacity of SS56-specific CTL. $SS56_{55-64}$-reactive CTL were isolated from PBL from a pSS patient by flow cytometry using HLA-A2/ $SS56_{55-64}$ dextramers (**a**). The isolated cells were expanded for 12 days using IL-2, phytohemagglutinin and a mix of irradiated lymphocytes from three healthy donors. The culture were examined in a chrome release assay in duplicates using either T2 cells without peptide, or pulsed with $SS56_{55-64}$ peptide or ML-$IAP_{280-289}$ peptide as target cells (**b**). Effector/Target ratio = 1:3, measurements were made in duplicates.

Clonotypes of $SS56_{55-64}$- and ML-$IAP_{280-289}$-isolated T cells

To extend this observation, we directly isolated both $SS56_{55-64}$-and ML-$IAP_{280-289}$-specific T cells from the same pSS patient by means of FACS to analyze them *ex vivo* (Figure 6a). In this respect, 98 $SS56_{55-64}$ and 114 ML-$IAP_{280-289}$-specific T cells isolated from 5×10^6 PBL were subjected to RT-PCR/denaturing gradient gel electrophoresis-based TCR clonotype mapping. Two T-cell clones were detected among the $SS56_{55-64}$-specific T cells (BV1 and BV4). Notably, one of these clones (BV4) was also present among the ML-$IAP_{280-289}$ reactive T cells; confirming that part of the $SS56_{55-64}$ T cells cross react with the ML-$IAP_{280-289}$ peptide (Figure 6b). The identity of the BV4 CDR3 regions was verified by sequencing (Figure 6c).

DISCUSSION

We recently described natural cellular immune responses against the inhibitor of apoptosis protein ML-IAP in melanoma patients. Thus, circulating cytotoxic T cells obtained from melanoma patients recognized an HLA-A2-restricted peptide ML-$IAP_{280-289}$ (QLCPICRAPV). However, this response could also be detected in a small fraction of normal donors (Andersen *et al.*, 2004b). T-cell receptors (TCR) recognize peptides complexed to HLA-molecules. Recognition of peptide/HLA ligands by the TCR is highly peptide specific, as minor variations in a T-cell epitope can completely change their capacity to induce T-cell activation. However, certain TCR can also recognize sequence-related and -unrelated (''mimicry'') epitopes presented by homologous MHC molecules. Hence, the TCR specificity repertoire is flexible in that the TCR can interact productively not only with wild-type peptide but also with slightly altered peptides (like partial TCR agonists or TCR antagonists) in the context of HLA-molecules (De Magistris *et al.*, 1992; Geluk *et al.*, 1997). This flexibility in TCR-mediated recognition of various peptide/MHC complexes may play a vital role in auto-immunity. The repertoire of peptide variants recognized is TCR clonotype dependent. ML-$IAP_{280-289}$ and $SS56_{55-64}$ are almost identical peptides. Hence, we examined if the ML-$IAP_{280-289}$-specific T cell cultures we had expanded from a melanoma patient cross-reacted with the $SS56_{55-64}$ peptide. We found that in the polyclonal ML-IAP-specific bulk culture there was

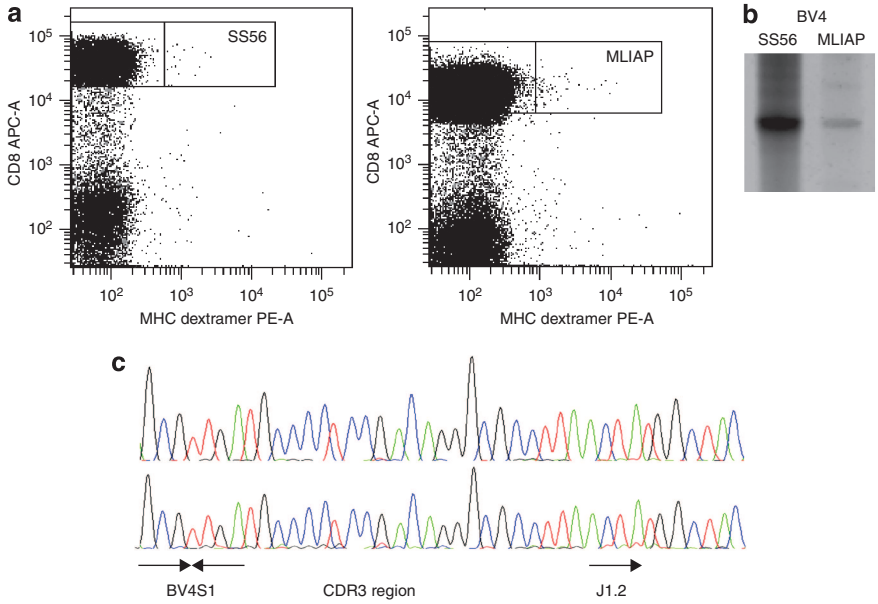

Figure 6. Clonotypes of $SS56_{55-64}$- and ML-$IAP_{280-289}$-isolated T cells from a pSS patient. $SS56_{55-64}$-specific T cells were isolated from PBL from a pSS patient by means of FACS using HLA-A2/ $SS56_{55-64}$ dextramers (**a**). The isolated cells were analyzed by the RT-PCR/denaturing gradient gel electrophoresis-based TCR clonotype mapping. This technique allows the analysis for T-cell clonality in complex cell populations, even if only small number of cells are available. As this method separates amplified TCR mRNA transcripts on the basis of the melting properties of the molecule, it is ideally suited for comparing TCR sequences, as identical molecules will resolve at an identical position in the gel. This revealed that the isolated cells consisted of two clones (BV1 and BV4). ML-$IAP_{280-289}$-specific cells were isolated from the same pSS patient using HLA-A2/ML-$IAP_{280-289}$ dextramers (**a**). The same BV4 clone could be detected among these cells (**b**). The identity of the BV4 CDR3 regions was verified by sequencing (**c**).

in addition some reactivity against the slightly altered peptide from SS56, indicating that it contained some T cells which cross-reacted with the slightly altered peptide from SS56. This was illustrated after cloning and expansion of specific cells, as we obtained clones highly specific for the ML-IAP, as well as cross-reactive clones. Peptide titration curves of the clones illustrated that a lower amount of ML-IAP peptide was needed for activation of the cross-reactive T cells compared to the SS56 peptide. These findings lead us to scrutinize if the $SS56_{55-64}$ peptide were indeed an auto-antigen for T cells in pSS and SLE. We describe that CTL responses against SS56 could readily be detected in blood and tissue samples obtained from pSS and SLE patients by means of flow cytometry and *in situ* detection assays using HLA-A2/ $SS56_{55-64}$ dextramers. The infiltration of SS56-specific T cells *in situ* suggests that these cells play a role in the pathogenesis of these diseases. Moreover, the functional capacity of the SS56-reactive cells was demonstrated by IFN-γ ELISPOT and cytotoxicity assays. It should be noted that the inclusion of other cytokines, for example, TNF-α ELISPOT, would have given a more precise description of the nature of the SS56-specific immune response, the phenotype of the specific cells, and would in addition have increased the chances for detecting T-cell responses, as some specific cells might release TNF-α and not IFN-γ. However, due to ethical reasons we were only allowed to draw a limited amount of blood from each patient.

Interestingly, the ELISPOT data shows a bimodal distribution. SLE is characterized by a wide range of clinical features and a prognosis that may range from benign to fatal. This heterogeneity among patients could, however, not be correlated with a CD8 response against SS56 in contrast to anti-SS56 antibodies, which have been associated with visceral complications in SLE (Billaut-Mulot *et al.*, 2001). However, it should be noted that when measuring HLA-restricted CTL responses it is always difficult to compare responders and non-responders. Hence, in patients without a detectable response there might be a response against another SS56 peptide restricted to the same or another HLA-molecule.

The identification of a target for CD8 T cells, which to our knowledge is previously unreported, may provide further insight into the cellular and molecular mechanisms of pathogenesis in two complex autoimmune diseases. Our findings suggest a role of CD8 auto-antigen-specific T cells in two diseases that have so far mainly been characterized by the presence of specific auto-antibodies. Although many studies have demonstrated a pivotal role of CD4 T cells in the induction and maintenance of the immune response (Namekawa *et al.*, 1995; Halse *et al.*, 1996; Helsloot and Sturgess, 1997; Davies *et al.*, 2002; Hoffman, 2004). The precise nature of T cell–B cell interactions are still to be fully delineated. In this regard, it would be very interesting to examine the possible correlation between anti-SS56 CD4, CD8 and antibody responses.

To further examine the cross reactivity between ML-IAP and SS56 reactive T-cell cultures we showed that ML-$IAP_{280-289}$-loaded target cells were lysed by $SS56_{55-64}$-specific T cells isolated from a pSS patient. In addition, we directly isolated $SS56_{55-64}$-specific T cells *ex vivo* by flow

cytometry and the subsequent TCR clonotype analysis revealed that these cells consisted of two clones. One of these clones could be detected among the T cells isolated from the same patient with the HLA-A2/ML-IAP$_{280-289}$ dextramer. Thus, SS56$_{55-64}$-specific T cells in this pSS patient cross reacts with the ML-IAP$_{280-289}$ peptide. The cross-reactivity between an epitope from a tumor antigen and an antigen involved in autoimmune disease accentuate the close relationship between autoimmunity and anti-cancer responses. Obviously, the involvement of peptide-specific CD8 T cells in SLE and pSS does not necessarily imply that this is the case in all autoimmune disorders. However, it does raise the question whether lessons could be learned regarding anti-tumor immune responses by studying autoimmune responses, in particular the mechanisms involved in regulating such responses. Counter-regulatory responses are important in the immune system; they help to limit the intensity and extent of immune responses, which otherwise could result in dangerous collateral damage to the host. However, with regard to anti-cancer immunotherapy, counter-regulatory responses antagonize the ability to create an intense immune response against the tumor. Moreover, this finding raises the question of whether potential cross-reactivity between a tumor antigen and other proteins should be considered when antigens are chosen for anti-cancer immunotherapy. This may be especially relevant when choosing the specificity of TCR in case of adoptive transfer with genetically modified lymphocytes as described in several studies (Morgan *et al.*, 2003, 2006; Kuball *et al.*, 2005, 2007).

Although it remains to be seen whether similar findings with other tumor antigens are exceptions or the rule, it is interesting to note that for quite a number of tumor antigens responses have been detected in a low proportion of normal donors. Renewed dialog between tumor immunologists, oncologists, and rheumatologists is required to compare and reconsider current models to delineate the similarities and differences between tumor and auto-immunity.

MATERIALS AND METHODS

Patients

Peripheral blood lymphocytes were collected from 20 patients with pSS and 20 patients with SLE from the Department of Rheumatology, Rigshospitalet, Copenhagen, Denmark; eight patients with pSS and 11 SLE patients were HLA-A2 positive. Furthermore, skin biopsies were obtained from 10 HLA-A2-positive and 10 HLA-A2-negative SLE patients with LE skin lesions. All pSS patients met the classification criteria for pSS (Vitali *et al.*, 2002). The auto-antibody status of the pSS patients was as follows: 18/20 positive for anti-SSA/Ro, 13/20 positive for anti-SSB/La, 17/20 positive for anti-ANA og 18/20 positive for anti-IgM-RF. The SLE patients were diagnosed according to the classification criteria for SLE (Tan *et al.*, 1982). The auto-antibody status of the SLE patients was as follows: 7/20 positive for anti-SSA/Ro, 2/20 positive for anti-SSB/La, 17/20 positive for anti-ANA og 12/20 positive for anti-DNA. The local Ethical Committee (Copenhagen, Denmark) approval was received for the study (number 01270846). The study is in accordance with the Helsinki declaration. Written informed consent was obtained from all participating patients. The study was also approved by the Danish Data Protection Agency (number 2005-54-1783).

Establishment of ML-IAP$_{280-289}$ T-cell clones

PBL from a melanoma patient were stimulated with irradiated autologous ML-IAP$_{280-289}$-loaded DC (PBL/DC ratio $= 3 \times 10^6$: 3×10^5) with IL-7 (5 ng ml^{-1}) and IL-12 (10 ng ml^{-1}) (PeproTech, London, UK). The cultures were restimulated ML-IAP$_{280-289}$-loaded irradiated autologous DC ($2\times$) followed by ML-IAP$_{280-289}$-loaded irradiated autologous PBL ($3\times$) and IL-2 (PeproTech, London, UK). PBL from a ML-IAP$_{280-289}$-specific culture were cloned by limiting dilution in the presence of a mix of irradiated lymphocytes from three healthy donors *ex vivo* with 5% heat-inactivated human serum, and IL-2 (PeproTech, London, UK). After expansion the clones were tested for specificity and cytotoxic potential in a standard ^{51}Cr-release assay.

INF-γ ELISPOT assay

To extend the sensitivity of the ELISPOT assay, PBL were stimulated once *in vitro* prior to analysis (McCutcheon *et al.*, 1997; Pass *et al.*, 1998). The ELISPOT assay was used to quantify peptide epitope-specific INF-γ releasing effector cells as described previously (Berke *et al.*, 2000). Briefly, nitrocellulose bottomed 96-well plates (Multi-Screen MAIP N45, Millipore, Hedehusene, Denmark) were coated with anti-IFN-γ antibody (Mabtech, Nacka Strand, Sweden). Stimulator T2 cells (with or without 10 μM peptide) and effector cells at different concentrations were added. Unpulsed T2 cells and T2 cells pulsed with HIV-1 pol$_{476-484}$ give similar responses in ELISPOT as described (Meier *et al.*, 2005). The following day, biotinylated secondary antibody (Mabtech) were added, followed by Avidin-enzyme conjugate (Mabtech) and finally the enzyme substrate NBT/BCIP (Mabtech). The reaction was terminated by washing with tap water on the appearance of dark purple spots. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers, LLC, Cleveland) and the peptide-specific CTL frequency could be calculated from the number of spot-forming cells. All assays were performed in duplicate for each peptide antigen.

Fluorescence-activated cell sorting (FACS)

PBL was analyzed by flow cytometry using FACSAria (BD Biosciences, Brøndby, Denmark). The T cells were stained with PE-conjugated MHC-Dextramers (DAKO, Glostrup, Denmark), followed by antibody staining with 7-AAD and the fluorochrome-couplet mAbs: CD8-APC and CD3-FITC (BD Biosciences, Brondby, Denmark). Both stainings were performed in RPMI-1640 media (GibcoBRL, Invitrogen, Taastrup, Denmark), for 20 minutes, 4 °C, in the dark. The MHC-Dextramer complexes used were: HLA-A2/ SS56$_{55-64}$ (YTCPLCRAPV), HLA-A2/ ML-IAP$_{280-289}$ (QLCPICRAPV) and as control HLA-A2/HIV-1 pol$_{476-484}$ (ILKEPVHGV). 100,000 CD8 cells were collected for each patient. CD8/ SS56$_{55-64}$ and CD8/ML-IAP$_{280-289}$-positive cells were sorted for TCR clonotype mapping.

Immunohistochemistry stainings

Multimerized peptide/HLA complexes were used to identify antigen-specific T cells *in situ* in lesions from SLE patients, as previously described (Andersen *et al.*, 2004b). The FITC-conjugated MHC-Dextramer complexes used were: HLA-A2/ SS56$_{55-64}$ (YTCPLCRAPV) and HLA-A2/ML-IAP$_{280-289}$ (QLCPICRAPV). Tissue sections

were dried overnight and subsequently fixed in cold acetone for 5 minutes. All incubation steps were performed in the dark at room temperature: (*a*) Forty-five minutes of the primary antibody (1:100 diluted) (*b*) Cy 3-conjugated goat antimouse (1:500 diluted; code 115-165-100; Jackson ImmunoResearch, obtained from Dianova, Hamburg, Germany) for forty-five minutes; and finally (*c*) the multimers for 75 minutes. Between each step, the slides were washed two times for 10 minutes in PBS/BSA 0.1%. The slides were mounted in vectashield and kept in the refrigerator until observed under the confocal microscope (Leica).

TCR clonotype mapping by denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis clonotype mapping of the human TCR BV regions 1–24 has been described in detail (thor Straten *et al.*, 1998). Briefly, RNA was isolated using the Purescript Isolation Kit (Gentra Systems Inc., Minneapolis, MN) and transcribed cDNA was amplified by PCR using primers for the variable regions of the TCR β chains in conjunction with a common constant region primer.

Cytotoxicity assay

CD8/SS56$_{55-64}$-specific cells were isolated and expanded shortly using irradiated lymphocytes from three healthy donors with phytohemagglutinin (Peprotech, London, UK) and IL-2 (Chiron, Ratigen, Germany). The expanded T cells were analyzed using a conventional 51Cr-release assay as described elsewhere (Andersen *et al.*, 1999). Target cells were peptide-pulsed, TAP-deficient T2 cells.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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